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Comparison of Human and Monkey Peptide Transporters: PEPT1 and PEPT2

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Abstract: Human proton-dependent peptide transporters, PEPT1 and PEPT2, mediate the cellular uptake of di- and tripeptides as well as a variety of drug molecules. Although PEPT1 and PEPT2 have been cloned from many species, there are no data available for monkey, an important pharmacological and preclinical species in drug development. In this study, it was first verified that monkey intestine transports a model dipeptide, Gly-Sar, in a proton-dependent manner (0.30 ± 0.05 pmol cm^{-2} s^{-1} at pH 6.0 and 0.10 ± 0.03 pmol cm^{-2} s^{-1} at pH 7.4) in the absorptive direction, presumably by monkey PEPT1. RT-PCR and rapid amplification of cDNA ends (RACE) were then used to clone monkey PEPT1 and PEPT2. Monkey PEPT1 (2127 bp and 708 amino acids) was found to be >94 and >92% identical to human PEPT1 at the cDNA and amino acid level, respectively. Monkey PEPT2 (2190 bp and 729 amino acids) was found to be >97% identical to human PEPT2 at both the cDNA and amino acid levels. Functional comparison of human and monkey peptide transporters expressed in HeLa cells suggested that functionalities of PEPT1 and PEPT2 were largely conserved in terms of Gly-Sar uptake kinetics and inhibitor specificity (for most tested substrates). Finally, Northern and RT-PCR analyses revealed some differences in tissue mRNA levels of peptide transporters between human and monkey.

Keywords: PEPT1; PEPT2; cloning; RT-PCR; RACE; species difference; monkey transporter

Introduction

In the process of drug discovery and development, species differences in drug biotransformation between preclinical animals and humans have been observed frequently.[1] It is of great interest to understand these differences at early stage of drug discovery and thus better predict and extrapolate data from animals to humans. In fact, significant effort is directed early in drug discovery to delineate species differences in metabolic and pharmacokinetic profiles of new drug candidates.[2]

In recent years, a large number of drug transporters have been cloned and identified in organs that are important for drug disposition, such as the small intestine, liver, kidney, and blood−brain barrier.[3] As the functions of these transporters are being characterized and rapidly documented in the literature, pharmaceutical researchers are beginning to gain an appreciation of the roles of drug transporters in determining absorption, distribution, metabolism, excretion, and toxicity (ADMET). Therefore, along with drug metabolic enzymes and other relevant factors, drug transporters should also be considered when addressing species differences on ADMET properties of drug molecules, especially when the

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(1) Grass, G. M.; Sinko, P. J. Physiologically-based pharmacokinetic simulation modeling. *Adv. Drug Delivery Rev.* **2002**, *54*, 433−451.

(2) Lin, J. H. Applications and Limitations of Interspecies Scaling and In Vitro Extrapolation in Pharmacokinetics. *Drug Metab. Dispos.* **1998**, *26*, 1202−1212.

(3) Kusuhara, H.; Sugiyama, Y. Role of transporters in the tissue-selective distribution and elimination of drugs: transporters in the liver, small intestine, brain and kidney. *J. Controlled Release* **2002**, *78*, 43−54.

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Table 1. Oligonucleotide Primers Used in RACE and RT-PCR

	cDNA	positions	sequence	product
RACE	PEPT1	782−808	5′-GGCTGGACTGGGCTAAAGAGAAATACG-3′	N/A
		808−782	5′-CGTATTTCTCTTTAGCCCAGTCCAGCC-3′	
	PEPT2	256−279	5′-CAGAGGCTGCTGAAGGCATGGTA-3′	N/A
		1940−1916	5′-TCTAGCATGAAATCTGTACTCCAGGC-3′	
RT-PCR	PEPT1	174−198	5′-GTTTGTGGCTCTGTGCTACCTGACG-3′	656 bp
		829−809	5′-ATGAGCGGCTCATCTCCCAAA-3′	
		7−32	5′-CCTTTCCAGAAAAATGAGTCCAAGGA-3′	411 bp
	PEPT2	417−394	5′-GCCTTACCAATACTGGGAGGACAA-3′	
		84−104	5′-CCCTCCAAAGAAGCCATCTCC-3′	821 bp
		904−883	5′-GATGTAAAGGCACTGACCAGGG-3′	

cellular entry and exit of these molecules are affected or governed by transporters.

The function and substrate specificity of drug transporters are often well characterized in human systems; however, such information in major preclinical species, especially in monkeys and dogs, is often lacking. This manuscript focuses on two proton-dependent dipeptide transporters, PEPT1 and PEPT2, which are among the most studied human drug transporters and significantly pharmaceutically relevant.[4−6] To be specific, PEPT1 has been actively investigated as a target for improving oral absorption of drug candidates, due to its high transport capacity in small intestine and its ability to accept a variety of peptide-based drugs as substrates. PEPT2 has been suggested as a potential target for drug delivery through pulmonary pathways.[7] In addition, PEPT2 has been suggested as a key renal transporter for reabsorption of β-lactam antibiotics and potentially other peptide-like drugs because of its high-affinity transport characteristics.

Although PEPT1 and PEPT2 have been cloned from many species, there are no data available for monkey, an important pharmacological and preclinical species in drug development. In this study, monkey PEPT1 and PEPT2 are cloned, and functional comparisons and tissue expression assessments are made between the monkey and human peptide transporters.

Experimental Section

Materials. Radiolabeled glycylsarcosine (Gly-Sar) (17 Ci/mmol for the ^{3}H-labeled form and 110 mCi/mmol for the [glycyl-1, 2-^{14}C] form) was purchased from Moravek Biochemicals, Inc. (Brea, CA). The BCA protein assay kit was purchased from Pierce (Rockford, IL). Unlabeled Gly-Sar, glycine, cefadroxil, captopril, cephalexin, enalapril, L-3,4-dihydroxyphenylalanine (L-DOPA), bestatin, and 5-aminolevulinic acid (ALA) were from Sigma (St. Louis, MO). The rabbit anti-human PEPT1 polyclonal antiserum was custom-made by Zymed Laboratories (South San Francisco, CA) and prepared from animals treated with a peptide corresponding to the last 16 amino acid residues of human PEPT1 (KSNPYFMSGANSQKQM). The rabbit anti-calnexin polyclonal antibody was from Stressgen (Victoria, BC). Anti-myc and anti-V5 antibodies were from Invitrogen (Carlsbad, CA). Human and rhesus monkey tissue total mRNA or RNA were from Biochain Institute (Hayward, CA). All oligonucleotides were synthesized at Sigma-Genosys (The Woodlands, TX).

Transport Experiments in the Ussing Chamber. The Ussing chamber assay was carried out using the intestinal tissue from adult male cynomolgus monkeys. Animal housing, care, and euthanasia were conducted in accordance with local animal care and use guidelines, and were approved by local committees. Six sheets per monkey ($n = 3$) of jejunum mucosa, stripped of one muscular layer, were mounted in Lucite Ussing chambers (0.625 cm^2 opening), which were connected to a model VCC MC6 voltage clamp apparatus (Physiological Instruments, San Diego, CA). The mucosal tissue was bathed in Ringer's with either 10 mM MES/Tris (pH 6.0) or 10 mM HEPES/Tris (pH 7.4), containing 10 mM mannitol and 100 μM Gly-Sar; the serosal solution was Ringer's with 2 mM mannitol, 10 mM glucose, 10 mM HEPES/Tris (pH 7.4), and 100 μM Gly-Sar. The volume of bathing solution on each side was 5 mL. After a 20 min equilibration period, [^{3}H]Gly-Sar was spiked into either the mucosal or serosal side of the chamber. Samples (1 mL) were taken from the unlabeled side in 15 min intervals for 3 h, and replenished with equal volumes of bathing solution. The tissue viability was assessed by a short circuit current (I_{sc}) jump after the addition of 100 μL of 1 M glucose to the mucosal side at the end of the experiment.

Cloning the Full-Length cDNAs of Monkey PEPT1 and PEPT2. RACE was performed with the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's protocols. Rhesus monkey small intestine and kidney poly(A)$^+$ RNA were used as templates to clone monkey PEPT1 and PEPT2, respectively. Oligonucleotides corresponding to highly conserved regions of

(4) Dantzig, A. H. Oral absorption of β-lactams by intestinal peptide transport proteins. *Adv. Drug Delivery Rev.* **1997**, *23*, 63−76.
(5) Rubio-Aliaga, I.; Daniel, H. Mammalian peptide transporters as targets for drug delivery. *Trends Pharmacol. Sci*. **2002**, *23*, 434−440.
(6) Herrera-Ruiz, D.; Knipp, G. T. Current perspectives on established and putative mammalian oligopeptide transporters. *J. Pharm. Sci*. **2003**, *92*, 691−714.
(7) Groneberg, D. A.; Nickolaus, M.; Springer, J.; Doring, F.; Daniel, H.; Fischer, A. Localization of the Peptide Transporter PEPT2 in the Lung: Implications for Pulmonary Oligopeptide Uptake. *Am. J. Pathol.* **2001**, *158*, 707−714.

PEPT2 (Table 1) from human, rabbit, rat, and mouse for PEPT1 and human, rat, and mouse for PEPT2 were used to amplify the 5′-end and 3′-end of monkey PEPT1 or PEPT2. The RACE products were cloned into the pCRII vector (Invitrogen) and sequenced. The full-length monkey transporters were generated from primers designed from extreme 5′- and 3′-ends of cDNAs using 5′-RACE-Ready cDNA as template.

For cloning of cynomolgus monkey PEPT1, total RNA was extracted from cynomolgus monkey small intestine tissue preserved in RNAlater stabilization reagent using RNeasy mini prep columns (Qiagen, Valencia, CA). A primer pair was designed from 5′- and 3′-UTR regions of rhesus monkey PEPT1, and was used to amplify the full-length cDNA sequence of cynomolgous PEPT1.

The open reading frames of the human PEPT1 and PEPT2 were first amplified from human small intestine and kidney QUICK-CLONE cDNA (Clontech) by *pfu* DNA polymerase (Stratagene, La Jolla, CA), respectively, and then subcloned into pCRII.

Construction of Expression Vectors for both Human and Monkey Transporters. The cDNA fragments of human and cynomolgus monkey PEPT1 were constructed to contain a *Bam*HI site at the 5′-side and an *Xba*I site at the 3′-side with or without the native stop codon. These fragments were then cloned into *Bam*HI−*Xba*I sites of pCDNA3.1/myc-His A (Invitrogen), resulting in four expression vectors, which encode either wild-type PEPT1 or a fusion PEPT1 with a C-terminal myc epitope and polyhistidine (His^6) tag. The cDNA fragments of human and rhesus monkey PEPT2 were directionally cloned into pcDNA3.1D/V5-His-TOPO (Invitrogen) according to the manufacturer's protocol, yielding four additional expression vectors, encoding wild-type PEPT2 and fusion PEPT2 with C-terminal V5 epitope and His^6 tag. Constructed vectors were verified by sequence analysis prior to functional analysis.

Transfection, Uptake Study, and Immunodetection of Human and Monkey PEPT1 and PEPT2 in HeLa Cells. HeLa cells were obtained from American Type Culture Collection (CCL-2). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4.5 g/L glucose, 110 mg/L sodium pyruvate, 584 mg/L L-glutamine, 0.1 mM nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO_2. After cells reached 70−80% confluence in 24-well plates, transfection was performed with FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. PEPT1- or PEPT2-mediated [^{14}C]Gly-Sar uptake activity was measured in the cells 24 h post-transfection. The uptake medium was at either pH 6.0 or 7.4 as described previously.[8] Nonspecific uptake due to passive diffusion was assessed in parallel experiments with cells transfected with the pcDNA3.1 empty vector. The incubation time for uptake experiments was 3 min, which is within the linear phase of the uptake process (the first 5 min). At the end of the incubation, the cells were washed three times with ice-cold PBS and lysed with 0.3 mL of PBS/well with 1% Triton X-100 for 30 min. The aliquots were subjected to both liquid scintillation and protein quantification by the BCA method. Uptake activity was determined as the number of picomoles or nanomoles of Gly-Sar per milligram of protein per 3 min.

To measure Gly-Sar transport kinetics, [^{14}C]Gly-Sar uptake in appropriate concentration ranges was assessed with an incubation time of 3 min. Passive diffusion (K_d, diffusion coefficient) was assessed in parallel experiments in HeLa cells transfected with an empty pcDNA3.1 vector. Experimental data were fitted by KaleidaGraph (Synergy Software, Reading, PA), in which a model describing the uptake as a process combining diffusion and single-site carrier-mediated transport[8] was used. The fitted kinetic parameters were presented as the maximal uptake velocity (V_{max}) and the concentration (K_t) when the uptake rate reaches half of V_{max}. All experiments were carried out in triplicate on two to three different experimental days.

For immunoblotting studies, the transfected HeLa cells in the six-well plate were washed with PBS and lysed with 0.5 mL of lysing buffer per well as described previously.[9] Lysates were then diluted and boiled for 5 min with an equal volume of 2× Laemmli sample loading buffer. Lysates were separated with a 4 to 20% SDS−polyacrylamide gel, and proteins were transferred onto an Immun-Blot PVDF membrane with a Criterion immunoblotting cell (Bio-Rad, Hercules, CA). The blot was probed with the appropriate antibody and visualized using a biotinylated anti-rabbit or anti-mouse IgG and a chromogenic detection system (Vector Lab, Burlingame, VA).

Northern Blot and RT-PCR Analysis. To analyze the tissue distribution pattern of rhesus monkey PEPT1 and PEPT2 mRNA, multiple-tissue poly(A)$^+$ RNA Northern blots (BioChain) were hybridized with [α-^{32}P]dCTP (3000 Ci/mmol; ICN Radiochemicals) probes prepared from cDNA fragments of monkey PEPT1 (nucleotide positions 745−1557) and PEPT2 (nucleotide positions 84−904) using a random primer DNA labeling system (Invitrogen). Hybridization was carried out in ULTRAhyb (Ambion, Austin, TX) at 50 °C overnight, followed by three washes at 55 °C in 2× SSC buffer containing 0.1% SDS and 1 mM EDTA. Blots were stripped with boiling 1% SDS to remove radiolabeled probe and reprobed with β-actin cDNA as an internal control. The blots were exposed to Bio-Max MR film or a PhosphorImager screen to visualize the signals.

Semiquantitative RT-PCR was performed on 1 μg of total mRNA (Biochain) with a SuperScript first-stand synthesis

(8) Liang, R.; Fei, Y.-J.; Prasad, P. D.; Ramamoorthy, S.; Han, H.; Yang-Feng, T. L.; Hediger, M. A.; Ganapathy, V.; Leibach, F. H. Human Intestinal H$^+$/Peptide Cotransporter. *J. Biol. Chem.* **1995**, *270*, 6456−6463.

(9) Wong, M. H.; Oelkers, P.; Dawson, P. A. Identification of a mutation in the ileal sodium-dependent bile acid transporter gene that abolishes transport activity. *J. Biol. Chem.* **1995**, *270*, 27228−27234.

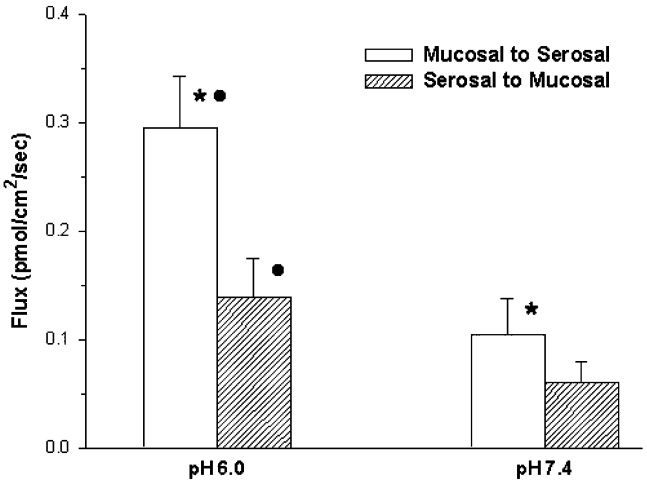

Figure 1. Proton-dependent mucosal-to-serosal Gly-Sar transport across monkey jejunum. The mucosal pH varied between 6.0 and 7.4 with the serosal pH constant at 7.4. [^{3}H]-Gly-Sar was spiked into either the mucosal or serosal chamber, each of which contained 100 μM unlabeled Gly-Sar. The pair of mucosal-to-serosal fluxes (indicated with asterisks) are different, as are the mucosal-to-serosal and serosal-to-mucosal fluxes (indicated with circles). Results are the mean ± the standard error from three to six tissues. Data were analyzed using the Student's *t* test, and a p of <0.05 was considered statistically significant.

system and Platinum PCR superMix (Invitrogen). PCRs were performed with PEPT1- or PEPT2-specific oligonucleotide primers (Table 1) with 35 cycles of denaturing at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 min, and the final extension was performed at 72 °C for 5 min. Amplified PCR fragments were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized by UV illumination. The PCR products were confirmed by sequencing the subcloned fragments.

Results

Proton-Dependent Absorptive Transport of Gly-Sar in Cynomolgus Monkey Jejunum. In the Ussing chamber study, the integrity of the jejunum tissue from cynomolgus monkey was assessed throughout the experiment by tissue resistance (R_t). These determinations showed that R_t remained constant ($\sim$40 Ωcm^2) over the time course of the experiments. This value is in good agreement with that previously reported.[10] When the pH of the mucosal bathing solution was changed from 7.4 to 6.0, the Gly-Sar mucosal-to-serosal flux increased significantly from 0.10 ± 0.03 to 0.30 ± 0.05 pmol cm^{-2} s^{-1} ($p < 0.05$, Figure 1). In addition, when the mucosal pH was 6.0, the mucosal-to-serosal (0.30 ± 0.05 pmol cm^{-2} s^{-1}) flux was significantly higher than the serosal-to-mucosal (0.14 ± 0.04 pmol cm^{-2} s^{-1}) flux ($p < 0.05$). In contrast, when the mucosal pH was 7.4, these two fluxes were not statistically different. Taken together, these data indicated that there is a proton-dependent dipeptide transport process across monkey small intestine in the absorptive direction, i.e., mucosal to serosal.

Cloning of Full-Length cDNAs of Monkey PEPT1 and PEPT2. Rhesus monkey PEPT1 and PEPT2 cloned by RACE were 3108 and 3227 bp long, respectively. The cDNA of cynomolgus PEPT1 was found to be 99.3% identical to that of rhesus PEPT1. The sequences have been submitted to GenBank (accession numbers AY289934 for rhesus PEPT1, AY289936 for cynomolgus PEPT1, and AY289935 for rhesus PEPT2). The cDNA sequences of cynomolgus and rhesus PEPT1 are 94.9 and 94.8% identical to that of human PEPT1 (GenBank accession number NM_005073), respectively. The level of cDNA identity between rhesus and human PEPT2 (GenBank accession number NM_021082) is 97.8%. The predicted amino acid sequences of the coding regions yield a 708-amino acid polypeptide for both cynomolgus and rhesus monkey PEPT1 and a 729-amino acid polypeptide for rhesus monkey PEPT2. There is a high degree of sequence homology between human and monkey peptide transporters at the primary amino acid sequence levels (Figure 2). Being 99.0% identical to each other, cynomolgus PEPT1 and rhesus PEPT1 are 92.7 and 92.4% identical to human PEPT1 at the amino acid level, respectively. The level of primary sequence identity between human and rhesus monkey PEPT2 is 97.0%.

Expression of Human and Monkey Peptide Transporters in Transfected HeLa Cells. Expression of human and cynomolgus PEPT1 and human and rhesus PEPT2 in transiently transfected HeLa cells was verified by Western blot analyses.

The anti-PEPT1 antibody was able to detect both human and monkey PEPT1 (75−100 kDa, Figure 3A). Since the anti-PEPT1 antibody used was raised against the 16-amino acid antigen in the human PEPT1 C-terminus, which is not identical to the corresponding region in monkey PEPT1 (Figure 2A), it is possible that this antibody may detect human PEPT1 with a higher efficiency than monkey PEPT1. It appeared that the level of immunoreactive human PEPT1 was indeed higher than that of monkey PEPT1, in comparison to the loading baseline provided by calnexin. Furthermore, both human and monkey C-terminally tagged PEPT1 could not be detected by this antibody, likely because the attachment of the myc epitope and His^6 tag to the C-terminus of PEPT1 blocks antigen−antibody interaction. When the antibody to the myc epitope was used in the Western blot analysis (Figure 3B), a comparable amount of human and monkey tagged PEPT1 was detected, suggesting that human and monkey PEPT1 are likely expressed at similar levels in the transiently transfected HeLa cells.

To verify that monkey PEPT1 is a proton-dependent cotransporter and to compare its transport function with that of human PEPT1, the uptake of the hydrolysis-resistant Gly-Sar dipeptide was assessed in mock-transfected cells with an empty vector and cells transfected with vectors containing PEPT1. The results (Figure 3C) showed that monkey PEPT1

(10) Wang, W.; Uzzau, S.; Goldblum, S.; Fasano, A. Human zonulin, a potential modulator of intestinal tight junctions. *J. Cell Sci.* **2000**, *113*, 4435−4440.

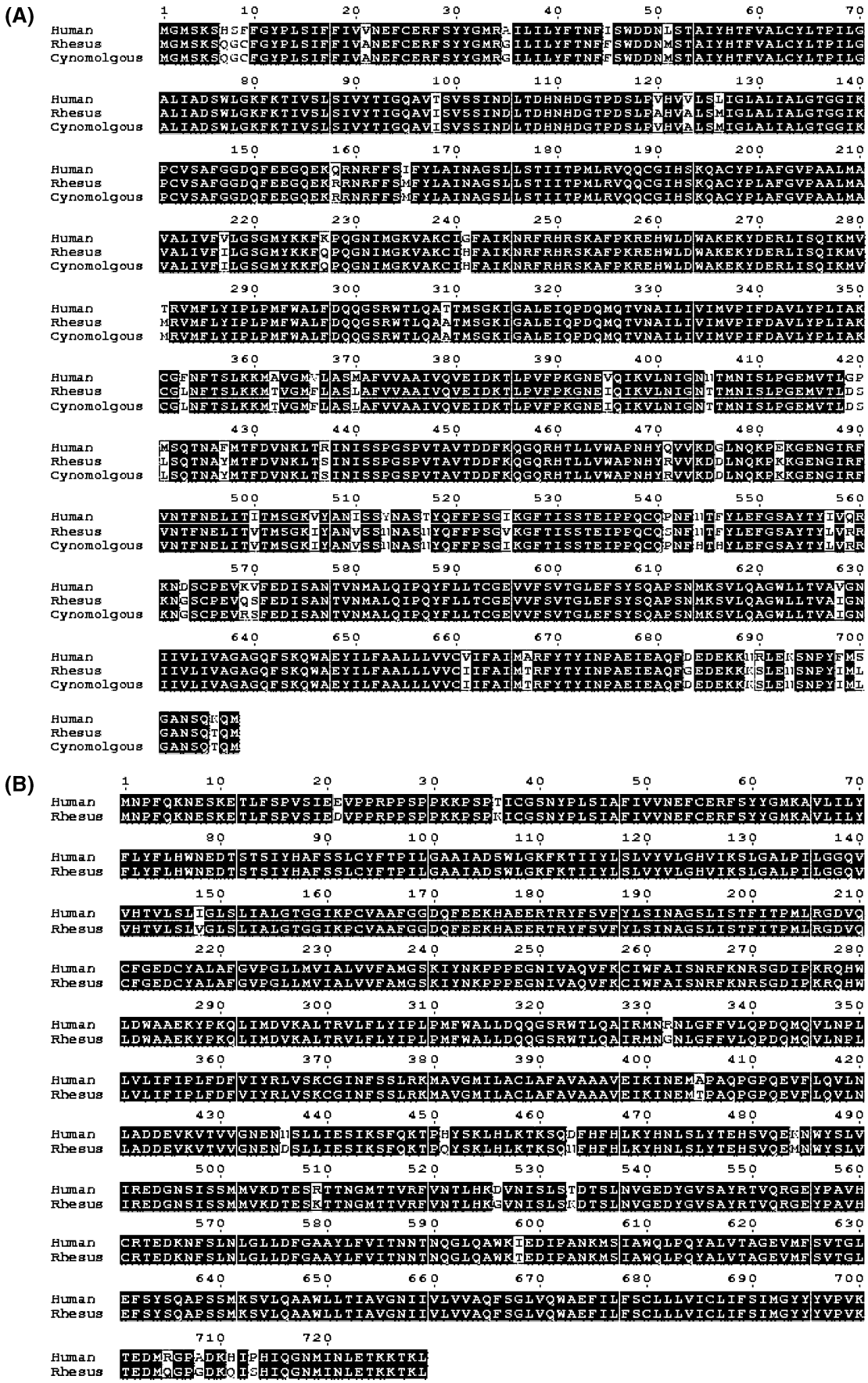

Figure 2. Primary sequence alignment of monkey and human peptide transporters: (A) PEPT1 and (B) PEPT2.

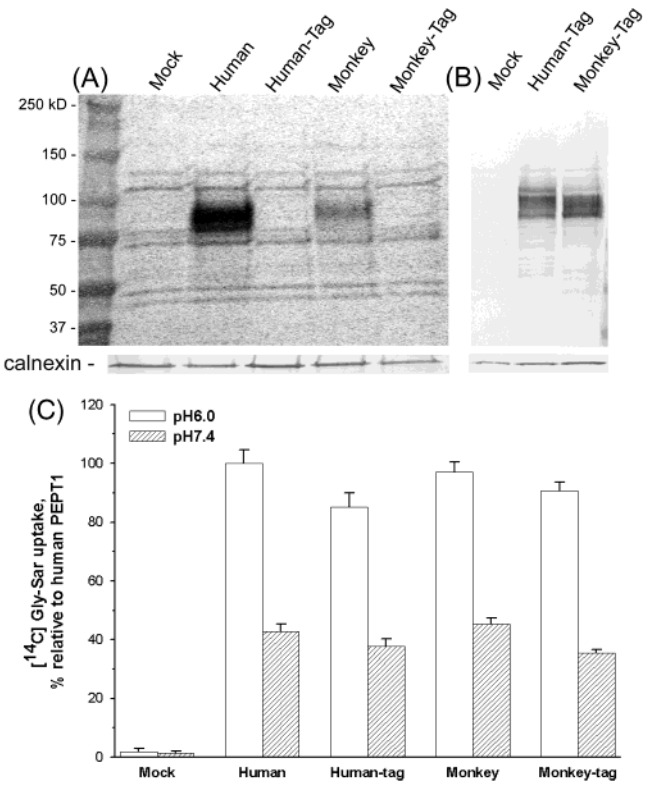

Figure 3. Expression of human and monkey PEPT1 in transiently transfected HeLa cells. (A) Proteins recognized by anti-human PEPT1 and anti-calnexin. (B) Proteins recognized by anti-myc and anti-calnexin. (C) pH-dependent Gly-Sar uptake by human and monkey PEPT1. HeLa cells were transfected with either empty vector (mock transfection) or PEPT1 with or without the myc His^6 tag for 24 h. Uptake of [^{14}C]Gly-Sar (50 μM) was assessed at pH 6.0 or 7.4 using a 3 min incubation time. Values are presented as percentages relative to the uptake value [2109 $\pm$ 99 pmol (mg of protein)$^{-1}$ (3 min)$^{-1}$] of human untagged PEPT1 at pH 6.0 and are the mean $\pm$ the standard error of four to six determinations.

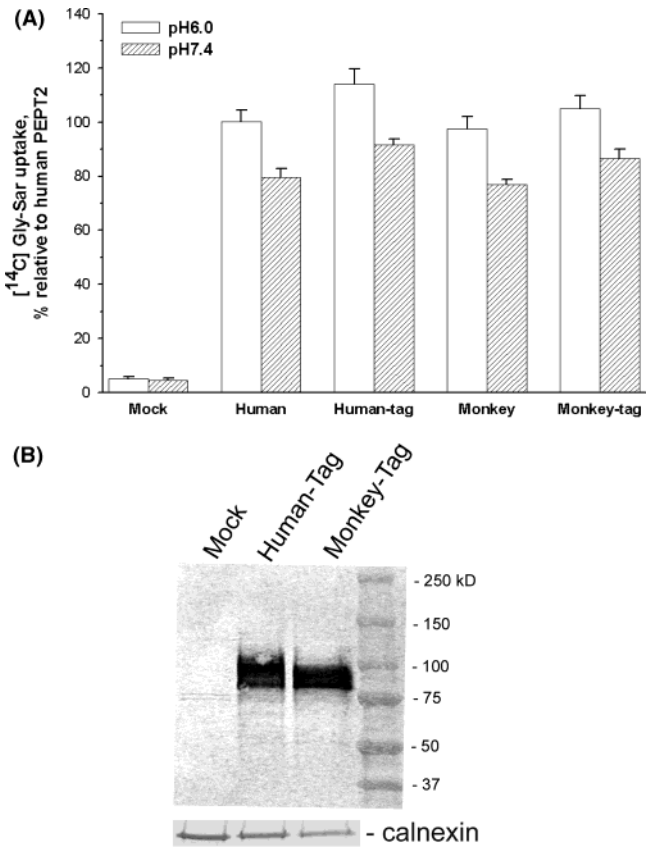

Figure 4. Expression of human and monkey PEPT2 in transiently transfected HeLa cells. (A) pH-dependent Gly-Sar uptake by human and monkey PEPT2. (B) Proteins recognized by anti-V5 and anti-calnexin. HeLa cells were transfected with either the empty vector or PEPT2 with or without the V5 His^6 tag for 24 h. The uptake of [^{14}C]Gly-Sar (30 μM) was assessed at pH 6.0 or 7.4 with an incubation period of 3 min. Values are presented as percentages relative to the uptake value [663 $\pm$ 34 pmol (mg protein)$^{-1}$ (3 min)$^{-1}$] of human untagged PEPT2 at pH 6.0 and are the mean $\pm$ the standard error of three determinations.

behaved in a manner similar to that of human PEPT1, as both transporters exhibited comparable uptake activities at either pH 6.0 or 7.4. Additionally, the attachment of the C-terminal tag (myc and His^6) to the transporters did not appear to alter their transport functions.

Similar results were obtained when transport activities were compared between human and monkey PEPT2 or tagged PEPT2 expressed in HeLa cells (Figure 4A). Monkey and human PEPT2 possessed almost identical Gly-Sar uptake under either pH condition, as did both tagged transporters. Additionally, the expression levels of both tagged transporters can be easily determined by the same antibody (antibody to V5 epitope) and were found to be comparable as shown in Figure 4B. Taken together, it appears that both human and monkey PEPT2 are likely expressed at comparable levels in transfected HeLa cells.

Comparison of Uptake Kinetics and Inhibitor Specificity of Monkey and Human Peptide Transporters. Uptake kinetics were determined at varying concentrations of Gly-Sar in the range of 0.039−5 mM for PEPT1 and 0.0039−0.5 mM for PEPT2. As shown in Figure 5A, both monkey and human PEPT1 revealed Michaelis−Menten-type saturation kinetics yielding similar kinetic parameters. Specifically, K_t and V_{max} equaled 0.39 $\pm$ 0.10 mM and 14.9 $\pm$ 2.2 nmol mg^{-1} (3 min)$^{-1}$ for human PEPT1 and 0.35 $\pm$ 0.18 mM and 11.3 $\pm$ 0.7 nmol mg^{-1} (3 min)$^{-1}$ for monkey PEPT1, respectively. For monkey and human PEPT2, their concentration−activity profiles were almost superimposable (Figure 5B) and yielded almost identical kinetic parameters: K_t and V_{max} values for human PEPT2 of 30.0 $\pm$ 5.4 μM and 1.6 $\pm$ 0.3 nmol mg^{-1} (3 min)$^{-1}$ and K_t and V_{max} values for monkey PEPT2 of 33.0 $\pm$ 4.8 μM and 1.6 $\pm$ 0.3 nmol mg^{-1} (3 min)$^{-1}$, respectively.

To further compare substrate specificity between human and monkey peptide transporters, competition experiments were performed for monkey and human peptide transporters. In these experiments, seven previously reported PEPT1 drug substrates[5] were used to test their relative inhibitory potential on Gly-Sar uptake for both PEPT1 and PEPT2 (Table 2).

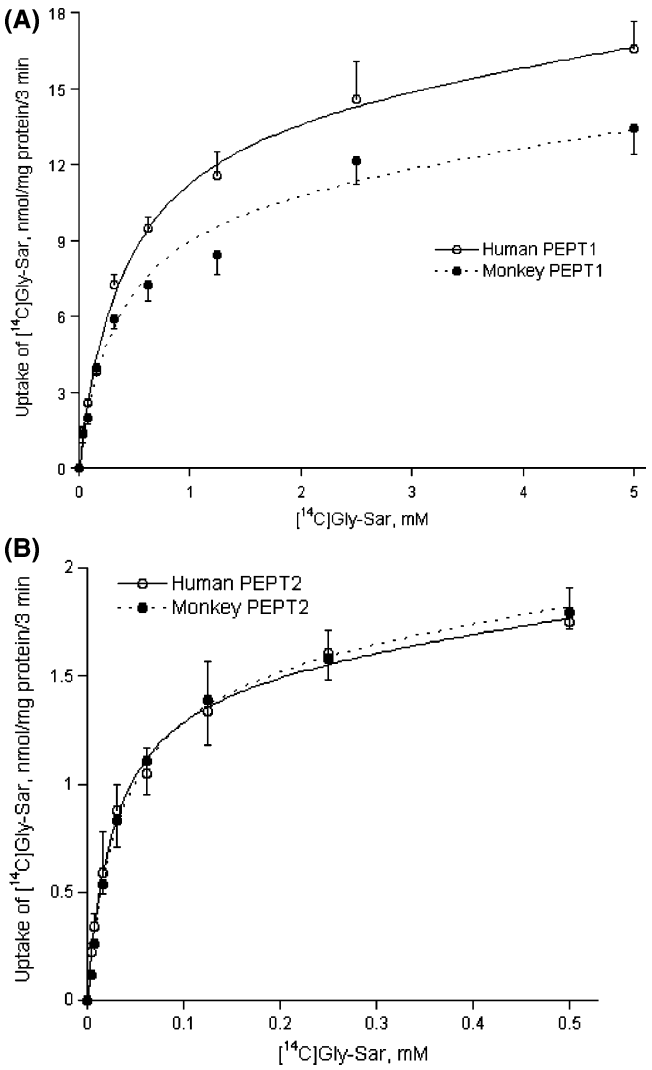

Figure 5. [^{14}C]Gly-Sar uptake kinetics of monkey and human (A) PEPT1 and (B) PEPT2. The uptake of [^{14}C]Gly-Sar by HeLa cells at pH 6.0 was assessed over concentration ranges of 0.039−5 mM for PEPT1 and 0.0039−0.5 mM for PEPT2. Values represent the mean ± the standard error of three determinations. The curves were fitted by a model containing both carrier-mediated and diffusion components (see the Experimental Section).

These drugs include β-lactam antibiotics (cefadroxil and cephalexin), angiotensin-converting enzyme (ACE) inhibitors (captopril and enalapril), peptidomimetic drugs (bestatin), and nonpeptidic substrates (L-DOPA and 5-aminolevulinic acid). The amino acid glycine was used as the negative control in the experiments and exhibited a minimal inhibitory effect on Gly-Sar uptake, while unlabeled Gly-Sar (positive control) diminished the uptake of [^{14}C]Gly-Sar as expected. The inhibition profiles of the seven compounds on Gly-Sar uptake were quite similar (demonstrated by the percentage of inhibition) in HeLa cells transfected with either human or monkey PEPT2. On the other hand, most compounds at the tested concentration exhibited a different ($p < 0.05$) inhibitory effect on [^{14}C]Gly-Sar uptake between human and monkey PEPT1. To more completely define the ability of these compounds to inhibit Gly-Sar transport, IC_{50} values were determined. The IC_{50} studies (Table 3) suggested that most of these compounds had comparable IC_{50} values for both human and monkey PEPT1. However, captopril and cefadroxil had slightly higher IC_{50} values for human PEPT1 than for monkey PEPT1 ($p < 0.05$).

Tissue Distribution of Monkey PEPT1 and PEPT2 mRNA. The tissue distribution of monkey PEPT1 and PEPT2 was examined by Northern blot analysis (Figure 6). A strong 3.4 kb transcript was detected in monkey small intestine. This band likely corresponds to monkey PEPT1 mRNA, since a 3.3 kb band for human PEPT1 mRNA was revealed by previous Northern analysis.[8] In contrast, no observable PEPT1 mRNA was detected in monkey brain, colon, kidney, lung, spleen, and skeletal muscle. Similarly, the 4.4 kb band in PEPT2 blot appears to correspond to monkey PEPT2, as PEPT2 mRNA of a similar size was previously reported in rabbits[11] and rats.[7] This 4.4 kb PEPT2 transcript was faint but detectable in monkey brain, kidney, and lung. When normalized with the β-actin expression level, the PEPT2 expression level was the highest in kidney, followed by that in the brain and lung. It should be noted that a 4.0 kb band in the PEPT1 blot and a diffused band around 3.5 kb in the PEPT2 blot were also detected from the same tissues where PEPT1 or PEPT2 transcripts were found, but their identities remain to be determined.

Semiquantitative RT-PCR was also performed for direct comparison of tissue expression of human and monkey peptide transporters in seven tissues, including brain, colon, kidney, liver, lung, pancreas, and small intestine (Figure 7). As a negative control, samples without prior reverse transcription yielded no detectable band (data not shown), suggesting that genomic DNA contamination was not the source of the amplified band. PEPT1- and PEPT2-specific amplicons were detected on all of the seven tested human and monkey tissues after 35 PCR cycles, but varied in band intensity. Visual comparison of these amplified bands indicates there were some differences in mRNA expression between human and monkey transporters in tissues. For example, the PEPT1-specific amplicon (656 bp) was barely detected from monkey kidney, but readily identifiable from human kidney. This suggests that the relative amount of PEPT1 mRNA in monkey kidney may be smaller than that of PEPT1 in human kidney. Similarly, PEPT2 mRNA in liver may be more abundant in monkey than in human, when the intensities of PEPT2-specific amplicons (411 bp) are compared. For PEPT2, two distinct PCR segments were generated from the total mRNA of monkey colon and small intestine. A similar observation was made with the other set of primers (see the sequence in Table 1; RT-PCR data not shown). The size of the shorter band was consistent with

(11) Boll, M.; Herget, M.; Wagener, M.; Weber, W. M.; Markovich, D.; Biber, J.; Clauss, W.; Murer, H.; Daniel, H. Expression cloning and functional characterization of the kidney cortex high-affinity proton-coupled peptide transporter. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 284−289.

Table 2. Inhibitor Specificity of Human and Monkey Peptide Transporters[a]

	PEPT				PEPT2			
	human		monkey		human		monkey	
inhibitor	pmol mg^{-1} $(3\ min)^{-1}$	%	pmol mg^{-1} $(3\ min)^{-1}$	%	pmol mg^{-1} $(3\ min)^{-1}$	%	pmol mg^{-1} $(3\ min)^{-1}$	%
none	1988 ± 85	0	1661 ± 68	0	631 ± 34	0	638 ± 37	0
Gly-Sar	76 ± 2	96	25 ± 13	98	125 ± 11	80	102 ± 16	84
glycine	1908 ± 61	4	1574 ± 29	5	637 ± 25	0	599 ± 19	6
ALA	199 ± 21	90	34 ± 6	98	226 ± 9	64	373 ± 26	58
bestatin	0	100	0	100	0	100	47 ± 7	94
captopril	775 ± 21	61	172 ± 26	90	592 ± 32	6	592 ± 18	7
cefadroxil	537 ± 43	73	202 ± 33	88	7 ± 2	99	4 ± 1	100
cephalexin	934 ± 32	53	522 ± 28	69	190 ± 15	70	165 ± 17	75
L-DOPA	1054 ± 27	47	653 ± 19	61	351 ± 23	44	280 ± 22	57
enalapril	258 ± 17	87	28 ± 14	98	152 ± 12	76	155 ± 9	76

[a] HeLa cells were transfected with vectors containing either PEPT1 or PEPT2 cDNA. The uptake of [^{14}C]Gly-Sar was assessed for 3 min at pH 6.0. The concentration of [^{14}C]Gly-Sar was 50 μM for PEPT1 and 30 μM for PEPT2. The concentration of the inhibitors was 10 mM for PEPT1 and 2 mM for PEPT2. Data were corrected for the uptake value of the mock-transfected cells. Values are the mean ± the standard error of four to six measurements. % is the percentage of inhibition.

Table 3. IC_{50} Values of Drug Substrates for Human and Monkey PEPT1[a]

	IC_{50}[b] (mM)	
inhibitor	human	monkey
ALA	0.78 ± 0.26	0.69 ± 0.21
bestatin	0.56 ± 0.19	0.74 ± 0.12
captopril	5.64 ± 0.81	2.61 ± 0.69[c]
cefadroxil	1.88 ± 0.35	1.05 ± 0.29[c]
cephalexin	11.1 ± 1.98	10.8 ± 2.30
L-DOPA	14.9 ± 2.45	13.7 ± 3.71
enalapril	2.13 ± 0.36	1.87 ± 0.21

[a] HeLa cells were transfected with vectors containing either human or monkey PEPT1 cDNA. The uptake of [^{14}C]Gly-Sar (10 μM) was assessed for 3 min at pH 6.0 in the presence of eight concentrations (between 0 and 50 mM, depending on the solubility) of inhibitors. IC_{50} values were determined by the dose−response IC_{50} model in KaleidaGraph. Values are represented as the average IC_{50} value ± the standard error of three independent experiments with three replicates per experiment. [b] The drug concentration that inhibits [^{14}C]Gly-Sar uptake by 50%. [c] Significantly different from that of human PEPT1 ($p < 0.05$).

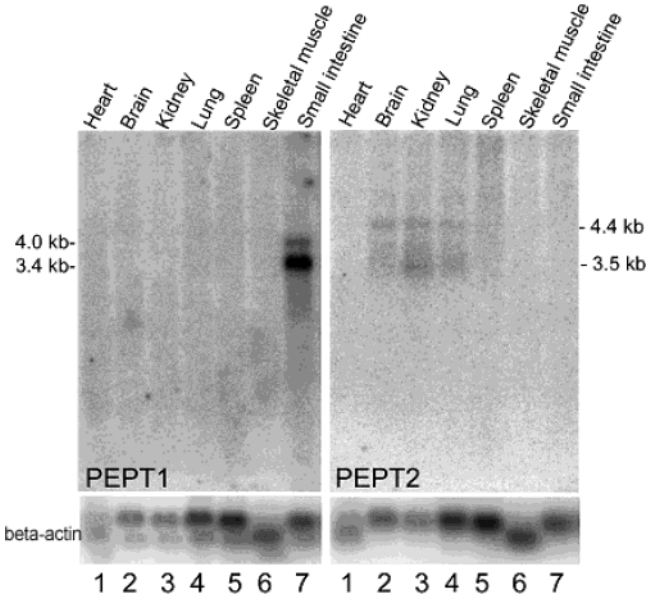

Figure 6. Tissue distribution of monkey PEPT1 and PEPT2 mRNA as examined by Northern blot analysis. Monkey multiple-tissue blots containing 3 μg of poly(A)$^+$ RNA were hybridized with radiolabeled probes prepared from full-length cDNA monkey PEPT1 or PEPT2 as described in the Experimental Section. The hybridized blots were stripped of residual radioactivity and reprobed with β-actin cDNA as an internal control. The blots were exposed to a PhosphorImager Screen for appropriate times to visualize the signals.

those from other tissues. Sequence analysis confirmed that this shorter transcript was indeed monkey PEPT2-specific and 411 bp in length, whereas the longer transcript was 600 bp in length and was identical to the wild-type transcript except for an 189 bp insertion.

Discussion

The objective of this study was to compare the transport function, substrate specificity, and tissue expression profile between human and monkey peptide transporters (PEPT1 and PEPT2). It should be noted that functional comparisons of PEPT1 were made between expressed cDNA from human and cynomolgous monkey, while that of PEPT2 was made between cDNA from human and rhesus monkey. The Ussing chamber work that demonstrated PEPT1 activity in small intestine was performed with cynomolgous tissue since cynomolgous monkey is the commonly used toxicology species. In addition, Northern and RT-PCR studies were carried out on RNA from rhesus monkey. It can be assumed that peptide transporters in these two monkey species should be quite similar (partially confirmed by the >99% level of sequence identity between cynomolgous and rhesus monkey PEPT1 in this study); therefore, the discussion of the results of these studies refers to monkey without further differentiation about which monkey species was utilized in a particular study.

Although no information was available in monkey, both PEPT1 and PEPT2 have been cloned from many species, including human,[8,12] mouse,[13,14] rabbit,[11,15] and rat,[16,17] and found to be highly homologous across species. The strategy adopted for cloning monkey PEPT1 and PEPT2 was based on this high degree of sequence homology. First, the most conserved regions on PEPT1 or PEPT2 cDNAs were deduced from cross-species sequence alignment; then primers were designed from these regions and used in RACE reactions to amplify monkey PEPT1- or PEPT2-specific 5′- and 3′-ends. Finally, the full-length clones of monkey PEPT1

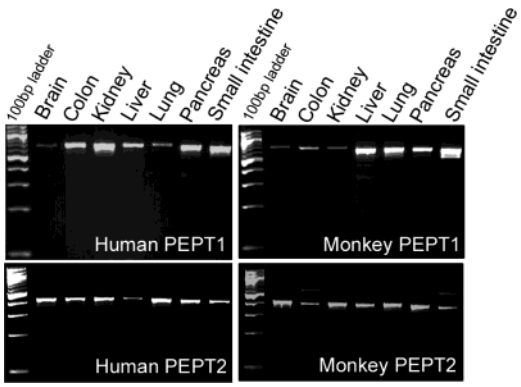

Figure 7. Tissue expression of human and monkey peptide transporters as examined by semiquantitative RT-PCR. The same loading (5 μL from 50 μL of the PCR mixture) per lane was analyzed by 2% agarose gel electrophoresis.

and PEPT2 were generated by PCR with a set of primers deduced from the sequence of the extreme 5′- and 3′-ends. Not surprisingly, both monkey peptide transporters exhibited a higher level of homology with human transporters than other species. As such, we recommend that this cloning approach should be applicable for cloning other monkey transporters.

To facilitate direct functional comparison of human and monkey peptide transporters, human and monkey PEPT1 were placed into the same expression vector pcDNA3.1 with and without the myc and His^6 tag. Similarly, human and monkey PEPT2 were placed into pcDNA3.1 with and without the V5 and His^6 tag. In fact, incorporation of a detection tag, such as GFP[18] and polyhistidine,[19] onto the N- or C-terminus of transporters has been proven to be a useful approach to studying the expression and function of many transporters in the heterologous expression system, provided that tagged transporters still retain function. A previous study demonstrated that the addition of the myc and His^6 tag to the C-terminus of human PEPT1 was tolerated well by PEPT1.[20] The results reported here further confirm that both human and monkey tagged PEPT1 and PEPT2 functioned in a manner similar to those of untagged transporters. Therefore, the expression levels of the untagged wild-type transporters could be reflected by that of the tagged transporters, whose expression was easily evaluated by Western blot using the commercially available antibody. This strategy eliminates the need to raise transporter-specific antibodies for cross-species detection which may be a problem due to amino acid variations in the antigen region (as in the case of PEPT1), or if an antibody is not readily available for the transporter (as in the case of PEPT2).

On the basis of the high level of primary sequence homology between human and monkey peptide transporters, it was anticipated that they would share similar transport function and substrate specificity. As a model dipeptide compound, Gly-Sar is a substrate for both human PEPT1 and PEPT2. Not surprisingly, Gly-Sar uptake kinetics were similar for human and monkey transporters (Figure 5). Subsequently, single concentrations (10 mM for PEPT1 and 2 mM for PEPT2) of several known substrates were used in the uptake inhibition study (Table 2). The percentages of inhibition of Gly-Sar uptake by tested compounds were similar in human and monkey PEPT2, whereas different inhibitory effects were observed with most compounds in human and monkey PEPT1. Since the tested concentration (10 mM) of some compounds led to nearly complete inhibition of Gly-Sar uptake by monkey PEPT1, IC_{50} studies were conducted to further evaluate the inhibitor specificity between human and monkey PEPT1. Most drug substrates possessed comparable IC_{50} values in human and monkey PEPT1. Captopril and cefadroxil, on the other hand, had lower IC_{50} values in monkey, which suggests they have greater affinity for monkey PEPT1 than for human PEPT1.

While functional comparison was performed in the heterologous expression system, in which peptide transporters were transiently expressed at a high level on the nonpolarized cell membrane, it appears from the results above that the monkey peptide transporter possessed a transport function similar to that of their human counterparts. In fact, Ussing chamber studies (Figure 1) with monkey mucosa showed that Gly-Sar was transported in a proton-dependent manner in the mucosal-to-serosal direction, presumably by the

(12) Liu, W.; Liang, R.; Ramamoorthy, S.; Fei, Y.-J.; Ganapathy, M. E.; Hediger, M. A.; Ganapathy, V.; Leibach, F. H. Molecular cloning of PEPT 2, a new member of the H^+/peptide cotransporter family, from human kidney. *Biochim. Biophys. Acta* **1995**, *1235*, 461−466.

(13) Fei, Y.-J.; Sugawara, M.; Liu, J.-C.; Li, H. W.; Ganapathy, V.; Ganapathy, M. E.; Leibach, F. H. cDNA structure, genomic organization, and promoter analysis of the mouse intestinal peptide transporter PEPT1. *Biochim. Biophys. Acta* **2000**, *1492*, 145−154.

(14) Rubio-Aliaga, I.; Boll, M.; Daniel, H. Cloning and Characterization of the Gene Encoding the Mouse Peptide Transporter PEPT2. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 734−741.

(15) Fei, Y. J.; Kanai, Y.; Nussberger, S.; Ganapathy, V.; Hediger, M. A. Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* **1994**, *368*, 563−566.

(16) Saito, H.; Okuda, M.; Terada, T.; Sasaki, S.; Inui, K. Cloning and characterization of a rat H^+/peptide cotransporter mediating absorption of β-lactam antibiotics in the intestine and kidney. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 1631−1637.

(17) Saito, H.; Terada, T.; Okuda, M.; Sasaki, S.; Inui, K. Molecular cloning and tissue distribution of rat peptide transporter PEPT2. *Biochim. Biophys. Acta* **1996**, *1280*, 173−177.

(18) Sun, A. Q.; Salkar, R.; Sachchidanand; Xu, S.; Zeng, L.; Zhou, M. M.; Suchy, F. J. A 14-Amino Acid Sequence with a β-Turn Structure Is Required for Apical Membrane Sorting of the Rat Ileal Bile Acid Transporter. *J. Biol. Chem.* **2003**, *278*, 4000−4009.

(19) Loo, T. W.; Clarke, D. M. The Minimum Functional Unit of Human P-glycoprotein Appears to be a Monomer. *J. Biol. Chem.* **1996**, *271*, 27488−27492.

(20) Theis, S.; Doring, F.; Daniel, H. Expression of the myc/His-Tagged Human Peptide Transporter hPEPT1 in Yeast for Protein Purification and Functional Analysis. *Protein Expression Purif.* **2001**, *22*, 436−442.

monkey PEPT1 transport system. Although direct comparison with human has not been performed, similar results were observed in different animal species.[21,22]

Tissue expression profiles of human and monkey peptide transporters were compared by both Northern and RT-PCR analyses. Previous Northern blot analyses showed that PEPT1 transcripts were readily detected in both small intestine and kidney in human,[8] rat,[23,24] and rabbit,[15] whereas our results (Figure 6) indicated that PEPT1 could be detected in only monkey small intestine. Since it has been generally accepted that both PEPT1 and PEPT2 are expressed in the kidney and are responsible for reabsorption of dipeptide and peptidomimetics, the absence of the PEPT1 transcript in monkey kidney by our Northern analysis was unexpected. Considering that Northern blot analysis may not be sufficiently sensitive to detect low-copy number transcripts, we performed RT-PCR, which indeed amplified a PEPT1-specific transcript from monkey kidney total mRNA at a high cycle number ($\geq$35 cycles). These results suggested that monkey kidney appears to contain a lower level of PEPT1 mRNA than human kidney. Furthermore, the PEPT1 mRNA level in colon also appeared to be higher in human than in monkey (Figure 7). That monkey PEPT2 mRNA was detected in kidney, brain, and lung by Northern blot analysis is in good agreement with the PEPT2 tissue localization reported previously in several other species.[11,23,25,26] The RT-PCR experiments reported here also demonstrated that both human and monkey PEPT2 mRNAs are present in the seven tissues that were tested, including small intestine. These results are inconsistent with previous reports which did not detect PEPT2 in small intestine by RT-PCR.[12,27] However, there are conflicting results from different laboratories on tissue expressions of PEPT1 or PEPT2 using RT-PCR. For example, human PEPT1 mRNA was detected in colon in one report,[28] but not in another.[29] A different RT-PCR protocol (specifically, different cycle numbers), different RNA and mRNA sources, and some intersubject variability may account for these inconsistencies.

Taken together, the results reported here indicated that there are some differences in mRNA tissue expression in human and monkey peptide transporters (i.e., a lower PEPT1 mRNA level in monkey kidney than in human). However, implications of these species differences remain to be determined, since the transcriptional mRNA level does not necessarily correlate to the active protein level.

Recently, a splice variant of human PEPT1 was cloned and shown to moderate the transport activity of human PEPT1.[11,30] Therefore, the observation of an extra PEPT2-specific amplicon in monkey colon and intestine (Figure 7) but not in human tissues by RT-PCR is an interesting finding. Preliminary splicing analysis suggested that this amplicon is an alternative spliced form of monkey PEPT2. Cloning and characterization of this potential PEPT2 variant will be the subject of future work.

In summary, PEPT1 was cloned from monkey small intestine and PEPT2 from monkey kidney, both of which were highly homologous in sequence to their human counterparts. In many respects, monkey peptide transporters, especially PEPT2, have a transport function very similar to that of their human counterparts. However, monkey PEPT1 appeared to confer higher affinity to two tested drug substrates, captopril and cefadroxil, than human PEPT1. In addition, tissue expression analyses on PEPT1 and PEPT2 mRNAs revealed differences between human and monkey. These differences between human and monkey peptide transporters will need to be considered when using monkey as a preclinical model to study drug disposition of the substrates of PEPT1 and PEPT2.

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(21) Winckler, C.; Breves, G.; Boll, M.; Daniel, H. Characteristics of dipeptide transport in pig jejunum in vitro. *J. Comp. Physiol., B* **1999**, *169*, 495−500.

(22) Hidalgo, I. J.; Ryan, F. M.; Marks, G. J.; Smith, P. L. pH-dependent transepithelial transport of cephalexin in rabbit intestinal mucosa. *Int. J. Pharm.* **1993**, *98*, 83−92.

(23) Shen, H.; Smith, D. E.; Yang, T.; Huang, Y. G.; Schnermann, J. B.; Brosius, F. C., III. Localization of PEPT1 and PEPT2 proton-coupled oligopeptide transporter mRNA and protein in rat kidney. *Am. J. Physiol.* **1999**, *276*, F658−F665.

(24) Miyamoto, K.; Shiraga, T.; Morita, K.; Yamamoto, H.; Haga, H.; Taketani, Y.; Tamai, I.; Sai, Y.; Tsuji, A.; Takeda, E. Sequence, tissue distribution and developmental changes in rat intestinal oligopeptide transporter. *Biochim. Biophys. Acta* **1996**, *1305*, 34−38.

(25) Groneberg, D. A.; Doring, F.; Nickolaus, M.; Daniel, H.; Fischer, A. Expression of PEPT2 peptide transporter mRNA and protein in glial cells of rat dorsal root ganglia. *Neurosci. Lett.* **2001**, *304*, 181−184.

(26) Groneberg, D. A.; Eynott, P. R.; Doring, F.; Thai Dinh, Q.; Oates, T.; Barnes, P. J.; Chung, K. F.; Daniel, H.; Fischer, A. Distribution and function of the peptide transporter PEPT2 in normal and cystic fibrosis human lung. *Thorax* **2002**, *57*, 55−60.

(27) Ganapathy, M. E.; Brandsch, M.; Prasad, P. D.; Ganapathy, V.; Leibach, F. H. Differential Recognition of β-Lactam Antibiotics by Intestinal and Renal Peptide Transporters, PEPT 1 and PEPT 2. *J. Biol. Chem.* **1995**, *270*, 25672−25677.

(28) Ford, D.; Howard, A.; Hirst, B. H. Expression of the peptide transporter hPepT1 in human colon: a potential route for colonic protein nitrogen and drug absorption. *Histochem. Cell Biol.* **2003**, *119*, 37−43.

(29) Merlin, D.; Si-Tahar, M.; Sitaraman, S.; Eastburn, K.; Williams, I.; Liu, X.; Hediger, M.; Madara, J. Colonic epithelial hPepT1 expression occurs in inflammatory bowel disease: Transport of bacterial peptides influences expression of MHC class 1 molecules. *Gastroenterology* **2001**, *120*, 1666−1679.

(30) Urtti, A.; Johns, S. J.; Sadee, W. Genomic Structure of Proton-Coupled Oligopeptide Transporter hPEPT1 and pH-Sensing Regulatory Splice Variant. *AAPS PharmSci* **2001**, *3*, article 6.